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MESSAGE:

To supplement the Amendment and Reply Under 37 C.F.R. § 1.116 filed October 28, 2005 with the U.S. Patent and Trademark Office, attached please find an *executed* copy of the Declaration Under 37 C.F.R. § 1.132, submitted *unexecuted* on October 28, 2005. The Appendices are not included with this facsimile transmission since they were previously filed with the U.S. Patent and Trademark Office on October 28, 2005.

CERTIFICATION

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No. 028622-0102

In re patent application of
Dorken, *et al.*

Group Art Unit: 1643

Serial No. 09/673,735

Examiner: P Tungaturthi

Filed: 12/27/2000

For: Novel CD19 x CD3 Specific Polypeptides and Uses Thereof

DECLARATION UNDER 37 CFR § 1.132

Commissioner for Patents
PO Box 1450
Alexandria, Virginia 22313-1450

We, Patrick A. Baeuerle and Peter Kufer, hereby declare:

1. I, Patrick A. Baeuerle, have worked in the field of immunology for almost twenty years. I have over 200 publications with most of the papers published in the field of immunology. I received a Ph.D. in 1987 from the University of Munich and I am presently a Professor of Immunology at the Ludwig-Maximilians University in Munich. I also serve as Chief Scientific Officer at Micromet. My curriculum vitae and a list of publications to further explain my experience and background is attached as **APPENDIX A.**

I, Peter Kufer, am an inventor on the present application. I have worked in the field of immunology for almost twenty years and have numerous publications in the field of immunology. I received a M.D. in 1991 and a Ph.D. in 1994 from the University of Munich. I am an affiliated lecturer with Ludwig-Maximilians University in Munich. I also serve as Vice President, Immunotherapy at Micromet. My curriculum vitae is attached as **APPENDIX B.**

2. We have read and understood the Office Action dated August 3, 2005, and particularly the Examiner's comments regarding Bohlen *et al.* (1993), Blattler *et al.* (1993), Mack *et al.* (1995), and the orientation of the claimed bispecific single chain (bsc) antibody (Ab) invention, namely V_LCD19-V_HCD19-V_HCD3-V_LCD3. We will first provide our understanding of the cited publications and later discuss the orientation and activity of the claimed invention.

Bohlen (1993) does not study the single chain bispecific CD19 x CD3 antibody. In addition, the article does not describe methods for the treatment of non-Hodgkin's lymphoma with an antibody.

Blattler (1993) describes the targeting of cells with toxic molecules, such as ricin. Toxic molecules are delivered to the cells by, for example, the use of antibodies that bind CD19. The invention requires the transport of the toxic molecule into the target cells. During this process the antibody used to target the cells is internalized together with the toxic molecule, thereby killing the cell. For example, in column 19, lines 20-51, Blattler discusses embodiments of his invention that include antibodies to the CD19 antigen covalently linked to a toxic molecule capable of binding and being transported into the target cell. Column 5, lines 23-52, further describes the previously unknown ability to separate the binding and translocation function of the two chains in the ricin molecule. Blattler shows how an antibody such as anti-CD19 can be used in conjunction with a portion of the ricin molecule to target and translocate into a target cell of interest. In stark contrast, the single-chain bispecific antibodies to be effective cancer treatments must not be internalized in order to effectively function as an anti-cancer agent recruiting tumor cells. See below, discussion of Mack.

Mack (1995) describes the use of a 17-1A x CD3 bispecific antibody construct (17-1A x CD3 bsc Ab). The potency of the 17-1A x CD3 bsc-Ab construct in redirected lysis of various tumor cells is not different from the potency of monoclonal antibodies described in the literature. (See Figures 5 and 6, page 7024). Half maximal target cell lysis is observed at concentrations of 17-1A x CD3 bsc-Ab between 1.6 (Kato cells) and 40 ng/ml (HT-29 cells). This low level of activity is achieved by whole antibodies as well, and is not attractive given the low productivity of 17-1A x CD3 bsc-Ab of only 12-15 mg/L in CHO cells (see page 7023). This particular bispecific antibody is not suited for clinical use because of the excessive costs associated with production and treatment. Either productivity of 17-1A x CD3 bsc-Ab requires dramatic improvement, or 17-1A x CD3 bsc-Ab needs to be more efficacious by at least 100 fold. Neither of these shortcomings is solved by Mack.

Mack proposes the use of 17-1A x CD3 bsc-Ab for the treatment of minimal residual disease as is characterized by a low burden of tumor cells and the presence of very small tumors. See p. 7025 and the discussion of 17-1A x CD3 bsc-Ab in the treatment of disseminated residual tumor cells after resection of the primary tumor. By contrast, the present

invention may be used in late-stage lymphoma, such as non-Hodgkin lymphoma (NHL). In NHL, massive amounts of tumor cells, sometimes in the kg-range, must be eliminated by a bispecific antibody. These tumor cells can float freely in peripheral blood or lymph nodes, but can also manifest as very large tumors. A bispecific antibody that can remove large tumor masses as found with certain blood-borne cancers is not described by Mack.

An antibody for the treatment of NHL must have a different specificity than the 17-1A x CD3 bsc-Ab because lymphoma cells are devoid of 17-1A antigen. The selection of an appropriate target antigen to address lymphoma is a challenging task. Although CD19 is very frequently expressed on human B cell malignancies and has previously been employed for immunotherapeutic approaches, CD19 is also known to rapidly internalize upon binding by monoclonal antibodies. See discussion above. While certain therapies, such as an antibody with a toxic molecule, seek to exploit internalization of CD19, internalization of CD19 in the case of bispecific antibodies would prevent recruitment of T cells and redirected lysis of tumor cells. As discussed below, the efficacy of the present invention relative to 17-1A x CD3 bsc-Ab on 17-IA expressing tumor cells and to other CD19/CD3 bispecific antibodies such as diabody, tandem diabody or quadroma is higher by 2-4 orders of magnitude.

3. In response to the Examiner's comments on pages 3-4 of the Office Action that the orientation of the claimed invention would be obvious over the cited scientific articles, we provide the following information which demonstrates that the claimed single-chain CD19 x CD3 antibodies have activity that could not have been predicted from the prior art. In fact, the prior art taught that bispecific single-chain CD3-C19 antibody "failed to recognize human CD3" (Kipriyanov *et al.*, The Fourteenth International Conference Adv. in the Applications of Monoclonal Antibodies in Clinical Oncology, p. 29 Thira Santorini, Greece (May 5-7, 1997) APPENDIX C). In the same report, Kipriyanov teaches making a non-covalent heterodimer diabody and reports this diabody "is potent in retargeting peripheral blood lymphocytes to lyse tumour cells expressing CD19 antigen." Our research group has conducted direct comparisons of the claimed bispecific single-chain CD19-CD3 antibody with a covalent heterodimer diabody and found that the claimed antibody has a vastly superior activity. A covalent heterodimer diabody was selected for comparative purposes because it has been shown to be more active than non-covalent heterodimer diabodies (see below). Our research group compared side-by-side the

claimed bispecific single-chain antibody with a diabody (Tandab), a well-characterized, covalent heterodimer diabody having specificity for the B cell antigen CD19. The claimed bispecific single-chain antibody has significantly superior activity. In addition, we have prepared and tested multiple sequences with the structure of the claimed bispecific single-chain antibody and found them to have similar activity. These data verify the extraordinary efficacy of the claimed invention over the claimed scope of single-chain bispecific antibodies having the structure V_LCD19-V_HCD19-V_HCD3-V_LCD3.

4. The bsc CD19xCD3 Ab in the claimed orientation was produced by CHO cells and purified from cell culture supernatant. Multiple variants of bsc CD19xCD3, called MT103, MB2.1, MB4 and VH19 were used in the following experiments. See the attached sequences. These variants were produced using techniques described in the specification on pages 6-8 and throughout the Examples.

MT103: The bsc CD19xCD3 amino acid sequence is provided in Figure 8 of the application. MT103 includes the C-terminal His tag to assist in purification, but not the Flag-Tag provided in Figure 8.

MB2.1: V_HCD3 portion of MB2.1 has 4 amino acid substitutions in positions 1 (D->H), 2(I->V), 3 (K->Q), 24 (T->A) compared to MT103. The V_LCD3, V_HCD19, and V_LCD19 are identical to MT103.

MB4: V_HCD3 portion of MB4 has several amino acid substitutions (more than 30) compared to MT103. The V_LCD3, V_HCD19, and V_LCD19 are identical to MT103.

VH19: V_HCD19 portion of VH19 has one amino acid substitution in position 89 (E->N) compared to MT103 introducing a potential glycosylation site. V_LCD19, V_HCD3, and V_LCD3 are identical to MT103.

We compared the claimed construct to a well characterized covalent heterodimer diabody called Tandab. For a comparison of the domain structures in the disclosed antibodies, please see the attached Summary of Constructs. The amino acid sequence of Tandab is shown in Figure 1. Previous studies showed by direct side-by-side comparison that Tandab (called LL-Tandab in the citation below) was more active than non-covalent heterodimer diabodies. (Kipriyanov et al, (1999), *J Mol Biol* 293, 41-56 APPENDIX D). For example, Kipriyanov states on page 51 that "[t]he Tandabs were consistently more effective than the diabody for inducing T cell

proliferation in the presence of tumor cells and in effector cell retargeting." Tandab is one of the best documented and studied heterodimer diabodies in this area of study. Tandab is therefore a well documented benchmark by which to compare the claimed invention with other similar constructs.

Tandab was produced in *E. coli* using the pBAD expression system (Invitrogen, Carlsbad, CA). A coding region was synthesized and sequenced (DNA 2.0 Inc., Menlo Park, CA) with a protein sequence as deduced from descriptions of the LL-Tandab protein by Kipriyanov et al, (1999) *J Mol Biol* 293, 41-56 and Kipriyanov et al, (1998). *Int J Cancer* 77, 763-72 (APPENDIX E), including PelB signal peptide, c-myc epitope and 6×histidine tag coding regions. The synthesized cDNA was cloned into a modified pBAD/*Myc*-His vector with the ampicillin cassette substituted with a kanamycin cassette giving the construct pBAD-Tandab KAN. The construct was transformed into *E. coli* strain BL21-AI (Invitrogen) and a 50 mL culture (LB medium, 50 µg/mL kanamycin, 37°C, 260 rpm) of a single transformant grown overnight was used to inoculate 12×1 L shake flask cultures (LB medium, 50 µg/mL kanamycin). The cultures were incubated at 25°C and 220 rpm until an $OD_{600\ nm}$~0.5 was attained. Gene expression was induced by addition of 0.08% (w/v) L-arabinose (Sigma, Taufkirchen, Germany) and further incubation for approximately 15 hours. The cells were harvested by centrifugation at 5,000× g for 12 min and resuspended in 900 mL 1×D-PBS (Invitrogen).

Periplasmic proteins were extracted by sequential freezing in ethanol/dry ice and thawing in a 37°C warm water bath for a total of six cycles. The crude extract was centrifuged at 9.000×g for 20 min and Complete EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany) added according to the manufacturers protocol, filtered (0.2 µm) and chromatographed on a 11 mL ProSep Chelating I (Millipore, Schwalbach, Germany) metal affinity chromatography column, equilibrated with 20 mM sodium phosphate, 400 mM NaCl, pH 7.0. Using a flow rate of 3 mL/min, the column was washed with 7 volumes of 20 mM sodium phosphate, 400 mM NaCl, 50 mM imidazole, pH 7.0, and bound protein was eluted with the same buffer added imidazole to 500 mM. Eluted fractions containing Tandab were added EDTA to 10 mM, concentrated four-fold and subjected to gel filtration on a HiLoad 16/60 Superdex 200 column (Amersham Biosciences, Freiburg, Germany) equilibrated with 1x D-PBS and running at a flow rate of 1 mL/min. Absorbance at 280 and 254 nm and conductivity were used to monitor

the purification. All purification procedures were performed at 4°C. The purity of Tandab was assessed by non-reducing SDS-PAGE. The corrected extinction coefficient (Absorbance$_{280}$ = 1) used was 0.49 mg/mL.

Non-reducing and reducing SDS-PAGE were performed in NuPage 4-12% Bis-Tris gels (Invitrogen), and proteins were stained with SimplyBlue SafeStain (Invitrogen, Carlsbad, California, USA) and destained in ultra pure water. For Western analysis, proteins were transferred onto a nitrocellulose transfer membrane by electroblotting. For immunological detection, the membranes were incubated overnight at 4°C with a 1:3000 dilution of a mouse anti-myc primary antibody (Invitrogen) followed by incubation with a 1:3000 dilution of an alkaline phosphatase-conjugated goat anti-mouse IgG Fc secondary antibody (Sigma) for 3 hours at room temperature. Both incubations were carried out in 1×D-PBS, 0,01% (v/v) Tween20, 3% (w/v) BSA. Alkaline phosphatases were detected colorimetrically using BCIP/NBT liquid substrate (Sigma, Taufkirchen, Germany).

Bispecific functionality of the Tandab was analyzed via FACS using human B-leukemic cell line Nalm-6 or T-leukemic cell line HP-Ball for CD19 or CD3 specificity, respectively. In brief, approximately 10^5 cells were incubated with 50 µL of the Tandab preparation (1 and 10 µg/ml) for 50 min. on ice. After washing with PBS/10%FCS/0.05% sodium azide, the cells were incubated with 30 µL of 2 µg/mL Penta-His IgG (Qiagen, Hilden, Germany) in PBS/10%FCS/0.05% sodium azide for 40 min on ice. After a second wash, the cells were incubated with 30 µL of a R-phycoerythrin (PE)-labeled goat anti-mouse IgG (Jackson ImmunoResearch, Cambridgeshire, UK; 1:100 in PBS/10%FCS/0.05% sodium azide) for 40 min. on ice. The cells were then washed again and resuspended in 200 µL PBS/10%FCS/0.05% sodium azide. The relative fluorescence of stained cells was measured using a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA).

All cell lines were purchased from the 'Deutsche Sammlung von Mikroorganismen und Zelllinien' (DSMZ, Braunschweig, Germany). MEC-1 cells were cultured in Basal Iscove's Medium, (Biochrom, Berlin, Germany), supplemented with 10% heat-inactivated fetal calf serum (FCS) from Invitrogen, Heidelberg, Germany. Nalm-6 and Raji cells were cultured in RPMI 1640 phenol red free medium (Invitrogen), supplemented with 10% FCS. For the cytotoxicity assays, all target cells were co-cultured with effector cells in RPMI 1640 phenol red-free medium supplemented with 10% FCS.

Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll density gradient centrifugation from leukocytes reduction filters as provided by local blood donor centers (Munich, Germany). Erythrocytes were removed by incubation in erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 100 µM EDTA) for 15 minutes at room temperature. Cells were centrifuged for 5 minutes at 600x g. The supernatant containing the lysed erythrocytes was discarded and the pellet washed with PBS. Thrombocytes were removed in an additional centrifugation step for 15 minutes at 100x g. PBMCs were finally resuspended and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS until use.

CD3 positive T cells were isolated from PBMCs using the Human T Cell Enrichment Column Kit (R&D Systems) according to the manufacturer's instructions. T cells used without pre-stimulation were isolated shortly before setting up the cytotoxicity assay.

A 75 cm^2 culture flask was coated for 90 min at 37°C with 10 µg/mL of OKT3, purified from hybridoma supernatant, and 2 µg/mL of anti-CD28 (15E8, Chemicon, Hampshire, UK) antibodies diluted in PBS. Approximately $1.7x\ 10^7$ isolated T cells were resuspended in 10 mL of cell culture medium and activated for 4 days in the coated culture flask in presence of 30 U/mL of IL-2. After 4 days of incubation, T cells were pelleted and resuspended in 25 mL of fresh cell culture medium containing 30 U/mL of IL-2. T cell suspension was transferred in a new 75 cm^2 culture flask and further incubated for one another day.

Target cells (MEC-1, Nalm-6 and Raji cells) were labeled using the PKH-26 Red Fluorescent Cell Linker Kit (Sigma, Taufkirchen, Germany) according to the manufacturer's instructions. In brief, $5x\ 10^6$ target cells were washed with PBS and resuspended in 250 µL of Diluent C. Cells were then mixed with 2.5 µL of PKH26 dye diluted in 250 µL of Diluent C and incubated at room temperature for 3 minutes with gentle agitation. After incubation, the staining reaction was quenched by addition of 1 mL FCS. Labeled target cells were then washed twice with cell culture medium.

All cytotoxicity assays were performed with isolated T cells as effector cells (pre-stimulated or not) and target cells pre-labeled with the membrane dye PKH-26. Effector and target cells were co-incubated in 96-well round-bottom plates at an E:T ratio of 5:1 for MEC-1 and Nalm-6 cells and at an E:T ratio of 10:1 for Raji cells. Per well, a defined number of $1.5x\ 10^5$ T cells was used. For measurement of the concentration-dependent cell lysis, 10-fold serial

dilutions of MT103 or Tandab were added to the samples. Cells were incubated in a total volume of 200 µL for 18 hours in a 5% atmosphere at 37°C.

After incubation, cells were pelleted by centrifugation and washed with FACS buffer (PBS, 1% FCS, 0.05 Na_3N). Subsequently, cells were resuspended in 200 µL of FACS buffer containing 1 µg/mL of propidium iodide to distinguish live from dead cells. Cells were analyzed using a FACS Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany). Per sample, a total of 30,000 target cells were acquired. Data were evaluated using the Cell Quest™ software (Becton Dickinson).

5. Hexahistidine-containing proteins were purified by metal affinity chromatography from periplasmic extracts of transformed *E. coli* cells. Tandab was separated from other species in the imidazole eluate by gel filtration (Fig. 2A). A dominant protein peak of approximately 120 kDa molecular size was collected by size exclusion as fraction I. Fraction I showed upon non-reducing SDS-PAGE a single polypeptide band of 114 kDa (Fig. 2B). This protein was fully converted to a 56-kDa band upon reducing SDS-PAGE (data not shown). The 114-kDa protein reacted in Western blot with an anti-myc antibody (Fig. 3B), indicating that it contained the C-terminal c-myc tag built into the Tandab protein (see Fig. 1). The protein was (i) absent from *E. coli* cells not transformed with Tandab cDNA (data not shown), (ii) was purified by metal chelate affinity chromatography, (iii) immunoreacted with anti-myc antibody and (iv) had the reported molecular size and dimeric structure. It therefore showed all features expected for a soluble Tandab protein expressed in *E. coli* periplasm (Kipriyanov et al., 1999; Reusch et al., 2004 APPENDIX F).

The identity of the 114-kDa protein in fraction I with Tandab was further supported by binding studies using fluorescence-activated cell sorting (FACS). Purified Tandab showed bispecific binding to CD3 on human T leukemia cell line HP-Ball (Figs. 3A and 3B) and to CD19 as expressed on human pre-B lymphoma line Nalm-6 (Figs. 3C and 3D). Binding was dose-dependent and showed robust shifts in mean fluorescence intensity of reporter cells at a concentration of 10 µg/ml Tandab.

In order to confirm that our Tandab protein was of equivalent biological activity as the published Tandab we analyzed the efficacy of Tandab for redirected target cell lysis. We first tested in a FACS-based cytotoxicity assay its efficacy with freshly isolated, unstimulated human

T lymphocytes as effector cells. The FACS-based assay determined the ratio between propidium iodide positive and negative target cells, and produced cytotoxicity data with ED_{50} values comparable to those derived from ^{51}Cr release, caspase 3,7 activation or adenylate kinase release assays (data not shown). A recent study with Tandab investigating redirected lysis of malignant B cells from chronic lymphocytic leukemia patients (Reusch et al., 2004) suggested that Tandab is also active with unstimulated T cells as are present in PBMC cultures, while previous studies with Tandab and diabodies used T lymphocytes pre-stimulated in cell culture with recombinant IL-2 and anti-CD3 monoclonal antibody OKT-3 (Kipriyanov et al., 1999). We confirmed here that Tandab could redirect unstimulated peripheral T cells for lysis of three human B lymphoma/leukemia cell lines. Raji, MEC-1 and Nalm-6 were all lysed in a Tandab concentration-dependent fashion at half maximal concentrations of 1.07, 1.27 and 0.24 nM, respectively (Figs. 4A, 4B and 4C; Tab. 1). We used E:T ratios of 10:1 for Raji and of 5:1 for MEC-1 and Nalm-6 cells. During the 18-hour assay period, between 40 and 80% of target cells were lysed. Nalm-6 cells were most sensitive to lysis, while Raji cells were most resistant.

Under identical assay conditions, MT103 also caused redirected lysis of all three cell lines with unstimulated peripheral T cells. Efficacy of cell lysis was, however, much higher than for the Tandab molecule. Values for half-maximal target cell lysis by MT103 were 0.4, 1.7 and 0.2 pM for Raji, MEC-1 and Nalm-6 target cell lines, respectively (Fig. 4A to C). This corresponds to a 736-2,605 fold higher cytotoxic activity of MT103 (Tab. 1). A larger proportion of MEC-1 cells were lysed by MT103-redirected T cells than by Tandab-redirected T cells, while Raji and Nalm-6 cells were lysed to a similar extent by the two kinds of bispecific antibodies during the 18-h assay period. Three sequence variants of bsc CD19xCD3 showed similar ED_{50} values as MT103 for redirected lysis of Nalm-6 cells with unstimulated T cells (see below).

In the next experiment, cultured T lymphocytes were used as effector cells, which had been stimulated for 4 days with immobilized recombinant OKT-3 and a T cell co-stimulatory agonistic anti-CD28 antibody, and for one day with human IL-2 prior to the cytotoxicity assay. Under these conditions of optimal pre- and co-stimulation of T cells, Tandab now reached close to 100% lysis of Nalm-6 cells, more than 80% of MEC-1 cells and 60% of Raji cells (Figs. 5A to C). Despite an enhanced overall cell lysis, no improvement of ED_{50} values was observed. ED_{50} values were 10.2, 6.0 and 0.24 nM for Raji, MEC-1 and Nalm-6 target cells, respectively (Tab.

1). Rather, there was a clear reduction by 10 and 6 fold in Tandab efficacy with Raji and MEC-1 cells, respectively, while it was unchanged for Nalm-6 cells.

Under identical assay conditions, MT103 reached close to complete lysis by redirecting pre-stimulated peripheral T cells of both MEC-1 and Nalm-6 target cells, and 80% lysis of Raji cells (Figs. 5A to C). Efficacy (ED_{50} values) for redirected target cell lysis with pre-stimulated T cells was improved for MEC-1 and Nalm-6 cells, and somewhat reduced for Raji cells compared to values obtained with unstimulated T cells (Tab. 1). All ED_{50} values of MT103 were at a low pM to fM level. With pre-stimulated T cells, the superiority of MT103 over Tandab was even more pronounced. MT103 was more potent in redirected lysis than Tandab by a factor between 2,218- and 8,062-fold (Tab. 1).

6. The bioactivities of the bsc CD19xCD3 Ab variants were analyzed in a cytotoxicity assay based on calcein release. Cytotoxicity assays were performed with target cells (Nalm-6, human B lymphoid cell line) loaded with Calcein AM (Molecular Probes) using human peripheral blood mononuclear cells (PBMCs) as effector cells. For the labeling of target cells calcein was added in a final concentration of 10 µM to a suspension of $1.5x10^7$ Nalm-6 cells in 5ml medium (RPMI medium with 10% heat-inactivated FCS and 25 mM Hepes) and incubated for 30 min. at 37°C at 5% CO_2. Calcein-loaded Nalm-6 cells ($0.5x10^5$) and human PBMC ($5x10^5$) were incubated with different concentrations of anti-CD19 x anti-CD3 for 4 hrs at 37°C at 5% CO_2 in triplicates. Mixed target and effector cells without addition of bispecific single-chain antibodies were used as controls. Cells were incubated with Saponin (0.1% final concentration) for 15 min in dark at room temperature and Calcein release was measured using a Spectrofluorometer at 535 nM emission. Percentage specific cytotoxicity was calculated according to the following formula: [(Sample value - control) : (maximum lysis – control)] x 100.

Cytotoxic activities of all variants with amino acid substitutions in the anti CD3 (Figure 6) and anti CD19 portion (Figure 7) were comparable to MT103. Both assays were performed with unstimulated human PBMCs as effector cells and Nalm-6 cells as target cells. In Figure 6, bispecific scFv anti EpCAM x anti CD3 was used as negative control. MT103 was used as positive control in comparing the bsc CD19xCD3 Ab variants MB2.1 and MB 4, both containing amino acid substitutions in the V_HCD3. The ED_{50} value of MT103 was 0.065 ng/ml. The ED_{50} values for MB2.1 and MB4 were comparable in magnitude.

In Figure 7, MT103 was also used as a reference molecule in comparison with the bsc CD19xCD3 Ab variant VH19 containing one amino acid substitution in the V_HCD19. The ED_{50} value of MT103 was 0.024 ng/ml. A comparable, even slightly smaller ED_{50} value was obtained for VH19.

Comparable conditions were used in Fig. 4C showing cytotoxic activities of MT103 and Tandab in direct comparison. In Fig. 4C the assay was also performed with Nalm 6 as target cells and with unstimulated human effector cells. The ED_{50} value of MT103 was 0.0156 ng/ml or 0.3 pM. The bioactivities of MT103 obtained in the different experiments were comparable and in the same range of magnitude. In contrast, the ED_{50} value of the Tandab was 28.5 ng/ml or 231 pM.

7. These experiments directly compare bispecific single-chain antibodies with domain arrangement V_LCD19-V_HCD19-V_HCD3-V_LCD3 with other constructs for potency of redirected target cell lysis. V_LCD19-V_HCD19-V_HCD3-V_LCD3 was consistently more active than Tandab and, depending on target cell line and pre-treatment of T cells had ED_{50} values between three and four orders of magnitude improved over Tandab. Tandab was selected since previous studies showed by direct side-by-side comparison that Tandab was more active than CD19/CD3-bispecific diabodies. Accordingly, V_LCD19-V_HCD19-V_HCD3-V_LCD3 is also much more active than these two other well-characterized bispecific antibody formats. In addition, we have characterized multiple V_LCD19-V_HCD19-V_HCD3-V_LCD3 constructs and verified that they all have similar assay activity. These findings underscore the remarkable and unexpected efficacy of the claimed invention in comparison to other modes of antibody treatment.

8. We hereby declare further that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By: _____ Date: 4/11/2005
Dr. Patrick A. Baeuerle

By: _____ Date: 8/11/2005
Dr. Peter Kufer